The Role of Molecules in Understanding Molluscan Evolution

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ABSTRACT

Molluscs comprise one of the most species rich animal phyla and exhibit incredible diversity in anatomy, development, ecology, and life history. Despite the extensive fossil record of Mollusca, phylogenetic reconstruction within the phylum has been controversial because some of the classes are highly modified. For the same reason, it has been difficult to identify the sister group to molluscs. With the advent of molecular techniques, a new array of characters has become available. Initially, nuclear ribosomal small subunit (18S) data was widely used and found to be useful in placing Mollusca within the bilaterian alliance known as Lophotrochozoa. These data have thus far proven to be of little use, however, in identifying the likely sister group within Lophotrochozoa to Mollusca or the relationships among the molluscan classes. A few other genes, notably complete nuclear ribosomal large subunit (28S), may have potential in resolving these questions. In addition to the sequence data, developmental genes and mitochondrial gene order data have become available for several groups and show great promise for future phylogenetic studies of Mollusca.Here we provide an overview of the current status for the different types of molecular data and what direction research is taking in the study of molluscan evolution.

INTRODUCTION

Few, if any, invertebrate groups of animals have received as much study as Mollusca, the second largest animal phylum after Arthropoda. Cuvier (1795) used several molluscan taxa to

illustrate the use of internal characters (soft body parts) in comparative anatomy. Subsequently, he defined the phylum Mollusca in its contemporary acceptance (Cuvier, 1797). Molluscs are coelomate protostomes and their anatomy is characterized in most cases by a well-developed head, a ventral foot, and a mantle layer covering a visceral mass. The mantle glands secrete calcareous skeletal elements, which can be spicules or shells that enclose much of the body. Ctenidia (the respiratory organs) are plesiomorphically present in pairs. The feeding organ of molluscs is the radula; a chitinous structure with numerous curved teeth often used in rasping. This complex structure is considered one the strongest morphological synapomorphy of the Mollusca, although it has been secondarily lost in bivalves and derived members of other groups. Molluscan ontogeny involves spiral cleavage and, in many cases, a triphasic life cycle composed of trochophore, veliger, and adult.

Four main sources of data can be used to reconstruct the evolutionary history of Mollusca: 1) anatomy; 2) fossils; 3) molecular sequences; and 4) genomic characters. By genomic characters, we refer to rare genomic changes such as mitochondrial gene-order data and large insertions or deletions of DNA, etc., as well as secondary and tertiary structures of biomolecules, and gene expression patterns of developmental genes. The continued use and exploration of this latter type of data will likely enhance understanding of both the phylogenetic relationships among molluscs and the associations between genotype and phenotype in the different molluscan body plans. An ideal appreciation for molluscan evolution will come from the integration of all sources of evidence, including fossils and anatomy. Here, however, we concentrate on reviewing recent evidence stemming from advances in molecular biotechnology that enlightens the phylogenetic history of Mollusca. Specifically, this chapter reviews what molecular sequence data and genomic characters suggest about the phylogenetic position of Mollusca within Metazoa, the identity of the closest-living relatives of molluscs, and the relationships among the major groups of molluscs that have traditionally been called classes. We also discuss new directions that molecular techniques are taking research in molluscan evolution and phylogenetics.

The phylogenetic position of Mollusca within Metazoa

Morphological and developmental traits such as segmentation and spiral cleavage have often lead to what could be termed a “traditional” morphology-based view that Mollusca shares a relatively close phylogenetic association with annelids (including echiurans and pogonophorans), arthropods, onychophorans, sipunculans, and tardigrades (Schram, 1991; Nielsen et al., 1996; Nielsen, 2001). However, since the time of a major review on different aspects of molluscan evolution (Taylor, 1996), this general placement of molluscs in the metazoan tree has been challenged to a certain extent, on the basis of molecular data. Specifically, a large number of analyses of nuclear small subunit ribosomal DNA (18S) data conform to the idea that molluscs share a relatively recent history with other animals that possess a trochophore larval stage, such as annelids (including echiurans and pogonophorans) and sipunculids. However, 18S studies also suggest that molluscs are more closely related to ectoprocts, nemertines and *Xenoturbella*, as well as to groups whose members have a lophophore (brachiopods, phoronids and ectoprocts), than they are to arthropods, onychophorans and tardigrades (Halanych et al., 1995; Winnepenninckx et al., 1995; Kim et al., 1996; Winnepenninckx et al., 1996; Aguinaldo et al., 1997; Littlewood et al., 1998; Zrzavy et al., 1998; Ruiz-Trillo et al., 1999; Adoutte et al., 2000).

An early such study defined the clade stemming from the last common ancestor of molluscs, annelids, brachiopods, bryozoans, and phoronids as Lophotrochozoa (Halanych et al., 1995). A related phylogenetic hypothesis based on 18S data is that arthropods are members of a clade, Ecdysozoa, that includes all animals that molt their cuticles (Aguinaldo et al., 1997). Some confusion about the definition of Lophotrochozoa has arisen in the literature of metazoan phylogenetics where it is often applied to all or most non-ecdysozoan protostomes (Aguinaldo et al., 1997; Ruiz-Trillo et al., 1999; Adoutte et al., 2000; Peterson and Eernisse, 2001). However, a review of the many studies of metazoan phylogeny based on 18S reveals that little evidence exists for the assertion that Platyhelminthes (with or without Acoela), Gnathostomulida, Gastrotricha, or Rotifera, etc. are descended from the common ancestor upon which the definition of Lophotrochozoa is based. Relationships among Mollusca and the other lophotrochozoan taxa, however, are not clearly delineated by 18S data.

While 18S studies have provided important insights into animal phylogeny, they are based on a single set of nucleotide characters and must be assessed in light of other data sources. A number of genes have been suggested as potentially useful for revealing relationships among major metazoan groups, for instance Elongation Factor –1Alpha, RNA Polymerase II and large

subunit (28S) rDNA. However, at present, just 28S has been applied to a broad diversity of metazoan groups (Medina et al., 2001a; Mallatt and Winchell, 2002; Winchell et al., 2002). Like 18S data, 28S sequences appear to support the Ecdysozoa and Lophotrochozoa hypotheses by suggesting that molluscs are more closely related to annelids, brachiopods, echiurans, nemertines, phoronids and sipunculans, than they are to Arthropoda and its molting allies (Mallatt and Winchell, 2002).

Most studies using molecular data to investigate the phylogenetics of Metazoa have relied on comparisons of aligned sets of nucleotides or amino acids. Over time, better theoretical models of nucleotide evolution have been applied to the analysis of nucleotide and amino acid data. In addition, as computer speeds and the amounts of molecular data increase, the methods used to analyze them have become more sophisticated. Nevertheless, just as for morphological characters, well-documented limitations exist for nucleotide data. They are subject to homoplasy, as well as single-site insertions and deletions (indels), nucleotide biases, different substitution rates, site-dependant saturation and other problems. As a consequence there has been increasing interest in finding new nontraditional phylogenetic markers (Rokas and Holland, 2000). Advances in genome research have made it easier to identify large genomic changes (i.e. intron indels, ribosomal secondary structure, organelle gene order data) which are starting to be examined for their potential use for phylogeny (reviewed in Rokas and Holland, 2000). We will refer to these new types of markers as genomic signatures. If they do occur at a rate that is appropriate for phylogenetic divergences being addressed, then genomic signatures should be powerful phylogenetic characters that can readily be analyzed by the cladistic method of phylogenetic reconstruction (Manuel et al., 2000; Rokas and Holland, 2000). For instance, Hox genes and neural expression of horseradish peroxidase immonureactivity are consistent with the Ecdysozoa and Lophotrochozoa hypotheses (de Rosa et al., 1999; Haase et al., 2001).

Mitochondrial DNA gene order data also play a potentially important role in understanding the position of Mollusca within Metazoa. Animal mitochondria generally have a circular genome which usually is less than 20 kb in size, and is characterized by a highly conserved gene content (13 protein coding genes, 2 ribosomal genes, and 22 transfer RNA genes) (Westenholme, 1992; Boore, 1999). Gene rearrangements in animal mtDNA genomes occur at a much slower rate than single nucleotide substitutions, and models of evolution of its gene order are appearing in the literature (Boore, 2000). Additionally despite the few animal

lineages sampled, gene order data have already been useful in reconstructing broad animal phylogenetic relationships (Boore and Brown, 1998; Boore, 1999). As this chapter goes to print, there are 240 complete mitochondrial genomes available either in the literature or in GenBank. Most of these genomes are chordates (178), but a rapidly growing number represent invertebrate phyla, including eleven from molluscs, two from annelids, and three from brachiopods. There are also several partial mitochondrial genomes known that include gene order data. A comprehensive review of all published gene order datasets in molluscs is available at: (http://www.jgi.doe.gov/programs/comparative/MGA_Source_Guide.html).

Although the relatively small number of genomes limits how much one can infer from gene order data at this point, these data have contributed something to our understanding of metazoan phylogeny. For instance, gene order data supports a lophotrochozoan clade, including molluscs (Boore and Staton, 2002, K. Helfenbein, pers. comm.). Unfortunately, while gene order comparisons within phyla tend to be conservative (e.g. within vertebrates), a major exception is Mollusca (Boore and Brown, 1994a,b; Boore, 1999, Boore, unpublished). Major rearrangements are observed among all the molluscan mtDNA genomes available (discussed below). The rapid evolution of gene order in molluscs is particularly well illustrated by the recent finding of major rearrangements among mitochondrial coding genes in several species of the gastropod genus *Dendropoma* (Rawlings et al., 2001).

The sister group to Mollusca

Asking, “What is the sister group to Mollusca?” is predicated on the assumption that Mollusca is monophyletic. In fact, at present, molecular data have provided little evidence to support such an assertion. Ghiselin (1988) and Winnepenninckx and coworkers (1994, 1995) did pioneering work in molluscan systematics using 18S. These studies included few molluscan taxa (under 10) as part of a larger metazoan data set, which was enough to weakly recover monophyly of molluscs but not to address relationships between the different major lineages. Analyses of large 18S invertebrate data sets do not always find optimal trees that contain a monophyletic Mollusca (Winnepenninckx et al., 1996; Giribet and Wheeler, 1999; Giribet et al., 2000; Peterson and Eernisse, 2001). In fact, this lack of resolution is broadly true for all the lophotrochozoan groups. Initially authors suggested that the lack of lophotrochozoan resolution

in 18S phylogenies was due to poor taxon sampling. The addition of more taxa however has yet to resolve the relationships among the lophotrochozoan lineages (Adoutte et al., 2000). Adoutte et al (2000) have proposed that this is likely a reflection of a burst of rapid speciation in the Cambrian within three major bilaterian lineages. On the one hand, if this is the case, new data from other molecular markers will also probably fail to resolve lophotrochozoan relationships or accurately reveal the phylogenetic status of the phyla that comprise Lophotrochozoa. On the other hand, new molecular data may help elucidate some of those questions for which 18S has failed to provide insight. Despite the poor resolution of the available data, no molecular evidence strongly falsifies monophyly of molluscs. Additionally, historical work on morphology certainly suggests that Mollusca is monophyletic.

A number of potential sister groups to Mollusca have been suggested in print. One potential sister group is Sipuncula, a phylum of vermiform organisms, whose larval morphology at the 64-cell stage is similar to that seen in molluscs, i.e., the molluscan cross (Scheltema, 1993), although it should be noted that some annelids also exhibit the same developmental pattern (R. Jenner, pers. comm.). Combined analysis of 18S data and morphology reached a similar conclusion (Zrzavy, 1998). Partial mitochondrial genome data for a sipunculan, however, supports a phylogenetic affinity to annelids rather than to molluscs (Boore and Staton, 2002). In many instances, molluscs have been suggested to have a close association with annelids (Ghiselin, 1988), or an assemblage including both annelids and arthropods (Schram, 1991; Nielsen et al., 1996; Nielsen, 2001). Turbellarian flatworms have also been considered to be contenders as the sister group of molluscs (Salvini-Plawen, 1991). Potential synapomorphies, namely similarities in larval morphology and circulatory systems, have also been proposed for a grouping of Entoprocta plus Mollusca (Haszprunar, 1996). While 18S data falsify the hypotheses that Arthropoda or any flatworm clade are part of a sister group to Mollusca, they do not contradict a sister group relationship with any combination of the lophotrochozoan taxa, including Sipuncula, Annelida, or Entoprocta.

Mallat and Winchell (2002), using complete 28S sequences, obtained some support for the idea that brachiopods and/or phoronids may be the sister group to molluscs. Even though this is a hypothesis that has not been discussed much in the literature in recent years, early analysis of secondary structure of the 5.8S had also suggested this relationship (Hendriks et al., 1986). There is, therefore, reason to re-evaluate the anatomical traits that were utilized previously to unite

these groups. For instance, pores formed from mantle extensions into the shell are present in both phyla. These structures are known as caeca in brachiopods and the shell-bearing molluscs, and as aesthetes in polyplacophorans (Reindl et al., 1995). According to ultrastructural observations and immunocytochemistry, there is a high degree of resemblance between these organs (Reindl et al., 1995; Reindl et al., 1997). However, Reindl and co-workers attributed this similarity to convergence, due to similar function, rather than to a shared phylogenetic history. If additional molecular evidence shows these two groups to be sister taxa, this character may be homologous.

Relationships within Mollusca

It is generally agreed that there are seven primary extant molluscan groups, which are correspondingly designated as classes of the phylum Mollusca. These classes are: Aplacophora; Bivalvia; Cephalopoda, Gastropoda, Monoplacophora, Polyplacophora and Scaphopoda. As shown in Figure 1, a general consensus view holds that the uni- and bivalved groups, collectively termed Conchifera, constitute a clade with Monoplacophora as the earliest diverging lineage (Scheltema, 1993; Salvini-Plawen and Steiner, 1996; Haszprunar, 2000; Nielsen, 2001; Brusca and Brusca, 1990; Haszprunar, 1996). Within Conchifera, Gastropoda and Cephalopoda are thought to be sister groups. Competing hypotheses suggest that Scaphopoda is either the sister group to Bivalvia (Scheltema, 1996) or Gastropoda plus Cephalopoda (Lindber and Ponder, 1996; Haszprunar, 2000). Additional controversy exists about whether the aplacophorans are monophyletic (Ivanov, 1996; Scheltema, 1996) or a paraphyletic grade representing the two earliest diverging lineages within Mollusca (Figure 1). Polyplacophora is also thought to be a relatively early diverging within Mollusca, although it has alternatively been hypothesized as the sister group to Aplacophora or Conchifera (Figure 1).

Not surprisingly, most of the molecular data that have been brought to bear on these questions comes from 18S sequences. Nevertheless, taxon sampling for this gene is still limited for some groups. Therefore, strong conclusions are premature. Most studies up to present have focused in the relationships within each class, or a particular class and its suspected sister groups. Well-represented classes for 18S in GenBank are Gastropoda and Bivalvia, whereas the other classes have less than a handful or no sequences reported at present. Authors have evaluated the potential for phylogenetic reconstruction of a few other genes traditionally used in molecular

systematics. These studies have involved partial sequences of the 28S (Rosenberg et al., 1994; Rosenberg et al., 1997), and several mitochondrial genes such as 16S and cytochrome oxidase I (Bonnaud et al, 1994, 1997; Giribet and Wheeler, in press; Harasewych et al, 1997; Carlini and Graves, 1999; Thollesson, 2000; Wollscheid-Lengeling et al, 2001). These studies also suffer of poor representation of all major molluscan lineages. Complete 28S sequences have been shown to elucidate the relationships within at least one metazoan phylum (Medina et al., 2001), and similar success can be hoped for in the case of Mollusca.

Mitochondrial gene order comparisons among molluscan classes

The chiton *Katharina tunicata*, was one of the first mtDNA genomes sequenced (Boore, 1994a; Boore, 1994b). This genome shows more features in common with other metazoan genomes (Figure 2a, only lophotrochozoan genomes depicted). These observations suggest that *Katharina* has retained several features that were present in the ancestral bilaterian mitochondrial genome, which has been used to polarize the evolution of molluscan rearrangements. Partial sequence (11 kb) from the aplacophoran *Epimenia verrucosa* supports the primitive status of this class based on the number of gene junctions shared with *Katharina* (Boore, pers. com.). The only cephalopod in the database (*Loligo bleekeri*) shares some gene junctions with *Katharina* but several unique rearrangements are present (Figure 2b). A second cephalopod (*Nautilus* sp.) has been sequenced and although it is conservative in its gene order, it has some rearrangements relative to *Loligo* (Boore, pers. comm.). The scaphopod *Dentalium eboreum* is highly rearranged relative to the other molluscan genomes (Boore, pers. comm.). Gastropods have been the most widely sampled group so far, however the sampling has been biased toward the crown lineages (heterobranchs) and only a substantial partial sequence is available for a caenogastropod (*Littorina saxatilis*) (Figure 2b). *Littorina* (Wilding et al, 1999) exhibits a conservative gene order, whereas the heterobranch gastropods share a widely rearranged gene order (Hatzoglou et al., 1995; Terret et al., 1996; Yalazaki et al., 1997; Kurabayashi and Ueshima, 2000a; Kurabayashi and Ueshima, 2000b).

In the case of the bivalve genomes, not only are they highly rearranged, but also they have lost or kept additional gene copies, which is a rare event in metazoan mtDNA evolution.

The ATPase 8 (atp8) gene is lost in *Mytilus* (Hoffman et al., 1992), which has previously only been observed in nematodes (Okimoto et al., 1991), whereas *Crassostrea* has a second copy of the small subunit ribosomal gene (Kim et al., 1999) (Figure 2b). Bivalve mitochondrial genomes are also particularly interesting because some species exhibit a special mode of mtDNA inheritance in which both male and female mtDNAs are passed onto the offspring. Females usually have a unique mitochondrial genome, whereas males exhibit heteroplasmy. The male somatic tissue contains mainly female mtDNA, but the germline is characterized by a dominant male-type mtDNA (Sibinski et al., 1994; Zouros et al., 1994). The male and female mitochondrial genomes show up to 30% sequence divergence depending on the species, and they are distinct to the point that there are major rearrangements between the two genders (Okazaki and Ueshima, 2001). This gender associated heteroplasmy has been termed "doubly uniparental inheritance" (DUI) (Zouros et al., 1994). Initially identified in marine mussels of the family Mitylidae (Sibinski et al., 1994; Zouros et al., 1994), it was subsequently detected in the related freshwater mussels of the family Unionidae (Hoeh et al., 1996, Okasaki and Ueshima, 2001). Recently, DUI has been characterized from a venerid clam of genus *Tapes*, a phylogenetically divergent group of bivalves (Passamonti and Scali, 2001). Although other venerid clams have been examined and no heteroplasmy is present (Passamonti, pers. comm.), this finding suggests that this type of inheritance may have been present in the ancestral bivalve that gave rise to the clade containing pteriomorph and heterodont bivalves (Passamonti and Scali, 2001). Bivalve lineages that are considered to have diverged earlier than the pteriomorph/heterodont split should now be sampled for DUI, as well as other molluscan groups. It remains to be evaluated whether the occasional presence of DUI in bivalve lineages may be accountable for the high number of rearrangements observed in them. With the help of molecular approaches, we are beginning to understand this unique mode of inheritance, which could not be previously detected by other methods of observation. Molecules can therefore be considered ideal markers to understand not only phylogenetic relationships but also biological phenomena at the organismal level.

With sequencing capabilities becoming greater, there is a growing number of molluscan mtDNA genomes being sequenced (Collins et al., 2001; Grande et al., 2001; Medina et al., 2001b). When multiple mitochondrial genomes become available there will be a two-fold benefit for studies in molluscan molecular systematics. First, the gene order data being considered the product of rare genomic rearrangements will help establish major groups with confidence by

revealing conserved gene junctions that can be used as synapomorphies. Second, a more thorough traditional molecular phylogenetic analysis, between and within classes, will be possible. Analysis of concatenated protein and tRNA datasets has already been a fruitful undertaking in vertebrate groups (Macey et al., 1997; Miya et al., 2001) that will be applicable to investigate molluscan relationships.

rRNA secondary structure

Even though most molecular systematists are aware of the additional information available in secondary structure of ribosomal genes, most studies limit the use of this sort of data to the improvement of the alignment (Kjer, 1995; Hickson et al, 1996). In many cases there is no secondary structure model available for a group of interest or related taxa, which affects the assessment of nucleotide homology. Additionally, nuclear ribosomal genes (28S and 18S) have been more widely studied and larger datasets are available for the secondary structure (Van de Peer et al., 2000; De Rijk et al., 2001) than their mitochondrial counterparts (16S and 12S). There is a growing interest, however, to develop new methods to ease the comparison and assembly of secondary structure model of new taxa for phylogenetic reconstruction (Billoud et al., 2000; Hickson et al., 2000; Page 2000; Parsch et al., 2000). The only study that has incorporated secondary structure into phylogenetic reconstruction of molluscan relationships is that of Lydeard et al. (2000). Representatives from four classes were included in the study (Polyplacophora, Bivalvia, Gastropoda and Cephalopoda) and a consensus molluscan secondary structure was folded. The models developed for each taxon used are available at http://www.rna.icmb.utexas.edu/. Even though the number of taxa was limited, three structural characters were identified as informative for phylogenetic reconstruction and were coded as binary characters for a cladistic analysis. Besides the great number of 28S and 18S sequences appearing in the literature, new 16S and 12S data will become also available as a consequence of the increasing number of complete mitochondrial genomes being sequenced. With the new advances in secondary structure reconstruction, the use of this type of data should become a standard practice in phylogenetic reconstruction when analyzing ribosomal data. Thus, this is another example of the potential of genomic signatures for molluscan phylogeny.

Gene family evolution in molluscs

Although most molecular systematists have concentrated their efforts on both ribosomal genes and mitochondrial genes for their phylogenetic studies, other nuclear gene data are available that can potentially be used to address phylogenetic questions. Particular cases are discussed below.

Hemocyanin: This gene encodes for a blue copper protein that acts as an oxygen carrier freely dissolved in the hemoplymph and it has been studied in several molluscan taxa (reviewed in van Holde and Miller, 1995). Although hemocyanins are also present in arthropods, there seems to be no evidence of common ancestry (Burmester, 2001). Partial sequences were available for several species, but only until recently has the gene been completely sequenced in a cephalopod (*Octopus dofleini*) (Miller et al., 1998). One isoform has been sequenced for the gastropod *Haliotis tuberculata*, whereas a second isoform is partially sequenced in this species (Lieb et al., 2000). In *Octopus*, hemocyanin is comprised of ten subunits, which in turn contain seven oxygen binding functional units (FUs) (Miller et al., 1998). In *Haliotis*, this protein is a didecamer with eight FUs instead of seven (Lieb et al., 2000). It reveals interesting features that are only shared among the two molluscs when compared to related proteins from outgroup taxa. Namely the placement of some introns 1) a linker intron between each pair of FUs, and introns separating 2) the two exons of the signal peptide, 3) the signal peptide from the first FU, and 4) the two halves of the untranslanted region of the 3' end (3' UTR) (Figure 1, Lieb et al, 2001a). Other introns show no correspondence between the two taxa. A phylogenetic analysis including most FUs from *Octopus* and *Haliotis*, in addition to the few FUs available from other molluscs, shows that they are homologous units and that they are likely the result of ancient multiple duplication events before the cephalopod/gastropod divergence (Lieb et al., 2000, 2001a). The comparison of the gene structure from just these two species, combined with x-ray cristallography data (Cuff e al, 1998) and the 3-D electron microscopy (Meissner et al., 2000) has allowed for a fairly sophisticated analysis of the evolution of this protein in molluscs. This initial effort has suggested that hemocyanin may be a useful phylogenetic marker to understand phylogenetic relationships at the class level, and other taxa such as chitons and bivalves are now being sequenced (Lieb et al., 2001b; Markl et al., 2001). The studies of hemocyanin will not only give

insight into the relationships among different classes but also into gene family evolution in molluscs.

Arginine kinase (AK): This usually monomeric molecule is a member of the highly conserved protein family phosphagen (guanidino) kinases (PK). It catalyzes the reversible transfer of phosphate from a phosphagen (arginine in this case) to ADP:

$$\text{phosphoarginine} + Mg^{+2}ADP \quad \overset{AK}{\Leftrightarrow} \quad \text{arginine} + MG^{+2}ATP$$

In invertebrates, there are at least six phosphagen kinases as opposed to one in vertebrates (creatine kinase), arginine kinase being the most widely distributed (Suzuki et al, 1997b).
A slightly different comparative approach was undertaken to study the evolution of this protein in molluscs relative to the work on hemocyanins. In this case, a combination of direct protein sequencing and amplification from AK cDNAs was used to obtain the amino acid sequence for several molluscs: 1) a chiton (*Liolophura japonica*) and a gastropod (*Battilus cornutus*) (Suzuki et al., 1997a), 2) a clam (*Pseudocardium sachalinensis*) (Suzuki et al., 1998), 3) three cephalopods (*Nautilus pompilius, Octopus vulgaris* and *Sepioteuthis lessoniana*) (Suzuki et al., 2000a), and 4) two more gastropods (*Cellana grata* and *Aplysia kurodai*) (Suzuki et al., 2000b). These studies identified a gene duplication and fusion present so far only in heterodont bivalves (*Pseudocardium*, *Solen* and *Corbicula* – the two latter are unpublished sequences only discussed in a manuscript) (Suzuki et al., 2000a). Although this can be considered a synapomorphy (a genomic signature) for this group of bivalves, when any of the two domains is used for phylogenetic reconstruction, the topologies are conflicting with the traditional view by placing bivalves branching earlier than the chiton. The authors conclude that bivalves are the most basal mollusc lineage (Suzuki et al., 2000b). Their topology, however, can be artifactual due to a limited taxon sampling and faster rates in the bivalve lineage, which in turn results in saturation and consequently incorrect reconstructions.

One important aspect of protein functional studies is identifying the substrate recognition region. In the case of molluscan AKs, a four amino acid region was recognized by site-directed mutagenesis. Introduced mutations that altered the amino acid composition resulted in reduced substrate affinity (Suzuki et al., 2000a). This amino acid region is not conserved in the two-

domain AKs from clams, which suggests that bivalve AKs may have a different substrate binding system (Suzuki et al., 2000a).

A phylogenetic analysis of several phosphagen kinases suggests an early divergence of AKs in metazoans. Cnidarian AKs branching off early, followed by a protostome clade containing a well supported arthropod lineage and a lophotrochozoan clade (molluscs and annelids) also strongly supported (Figure 3, Suzuki et al, 1997b). We can conclude that Arginine kinase sequence data from three different molluscan classes and a few outgroups can have potential as a phylogenetic marker for molluscan systematics.

Shell proteins: The molluscan shell is a mineralized structure composed of layers of calcium carbonate crystals and organic polymers. There are two kinds of crystals, the prismatic layer formed of calcite and the nacreous layer formed of aragonite. It is suspected that the organic matrices secreted by the mantle play a critical role in shell formation. Consequently, several shell matrix proteins have been recently sequenced from different molluscs, and protein secretion has been characterized to be exclusive to the mantle. In gastropods, two distinct shell proteins are identified from the red abalone, *Haliotis rufescens* (Bowen and Tang, 1996; Shen et al., 1997) and several from the oyster pearl, *Pinctada fucata* (Miyamoto et al., 1996; Miyashita et al., 2000; Sudo et al., 1997). Some of these different extracellular matrix proteins are likely present in many molluscs. Thus, they could be used as traditional phylogenetic markers or could be examined for genomic signatures if the gene organization is determined.

MOLLUSCAN EVO-DEVO

Comprehensive reviews of molluscan comparative embryology from reproduction to organogenesis can be found in the contributions in Verdonk et al. (1983). For an updated summary, refer to van den Biggelaar et al (1994) where they describe the pros and cons of molluscs as developmental models. Here we intend to give some background information followed by a short overview of recent molecular findings.

The study of metazoan evolution has traditionally been based on comparative embryology, anatomy and paleontology. The development of both phylogenetic theory and molecular systematics were the first fundamental steps to revolutionize the field. However, the

most recent and rapid advances come from molecular developmental biology. New molecular tools finally allow the analysis of patterns of gene expression, but most importantly they are crucial in producing a better understanding of how different genes affect biological processes in the overall functioning of the cell and the organism. Differential gene expression both during development and in the adult stage is what makes an organism unique in phenotype and physiology, and use of these approaches in a comparative framework has produced a new synthesis in the study of animal body plan evolution. This is now known as the rapidly growing field of evolutionary developmental biology or evo-devo.

With the exception of cephalopods, molluscan development starts with spiral cleavage, followed by blastula and gastrula stages. Even though some groups exhibit direct development, most groups possess a lecithotrophic trochophore larval stage (Nielsen 2001). Gastropods and bivalves usually have a feeding veliger larval stage as well. Whereas larvae are mobile, adults are usually slow moving. Although both stages use the shell and the operculum as a protective shield, however, their feeding habits are modified according to their different living environments. Thus, the diverse life history strategies adopted by many species, combined with extreme body plan modifications, make molluscs an ideal group to investigate the plasticity of the underlying mechanisms of development. In the particular case of molluscs, evo-devo studies are in their infancy, yet a growing interest is already reflected in the recent literature.

The groundwork for modern developmental studies was laid at the turn of the 20th century when embryologists carefully described the early development of several spiralian taxa (Kofoid, 1894; Wierzejski, 1905; Smith, 1935). Later work involved embryological manipulations (i.e., deletion experiments) which helped unravel some of the mechanisms underlying spiralian development (Guerrier, 1970a,b,c). For instance, it was shown that the D quadrant acts (in particular the macromere 3D, and the mesentoblast or 4d), as an organizer of dorso-ventral patterning by carrying cytoplasmic determinants that signal developmental fates of other blastomeres (Clement, 1976; van den Biggelaar, 1977; Damen, 1994). Additionally, cell lineage studies demonstrated that most molluscan embryos usually develop in one of two different manners: 1) unequal cleavage (this includes polar lobe forming taxa) yields different size quadrants from the first cell division, and 2) equal cleavage produces similar size blastomeres in the first two cell divisions (Boyer and Henry, 1998; Freeman and Lundelius, 1992). In molluscan equal cleavers, cell fate is induced late by the D-quadrant, whereas blastomere specification occurs early in

unequal cleavers (Freeman and Lundelius, 1992). Due to the lack of a reliable phylogeny for lophotrochozoans, no strict character state reconstruction has actually been performed on developmental modes in spiralians, however, a loose attempt suggests that equal-cleavage with late specification is the ancestral state (Freeman and Lundelius, 1992). As cell division proceeds to later stages, cell lineages tend to differ between taxa (Freeman and Lundelius, 1992). By character mapping of cell lineages on an accepted gastropod phylogeny, it has become apparent that there seems to be an evolutionary trend towards an early formation of the mesentoblast in more derived lineages (van den Biggelaar, 1996; van den Biggelaar and Haszprunar, 1996). Guralnick and Lindberg (2001) presented the first attempt at a rigorous phylogenetic reconstruction of molluscan relationships based on cell lineage data. To be able to use cell lineages in a modern phylogenetic analysis (maximum parsimony and distance-based), they developed a relative measure of cell formation that allowed them to code homologous characters. Due to the limited taxon sampling and some homoplasy in the data set, the resulting topologies recovered only a few nodes. These data, however, suggest that gathering of more cell lineage data for molluscan phylogenetics may be a worthwhile effort.

Most of the work on molluscan development has been carried out with gastropod embryos, although development of taxa from some of the other classes has occasionally been described. Aplacophorans, despite their key taxonomic placement have been neglected. The development of two aplacophoran taxa, however, has recently been described by using light and scanning electron microscopy (SEM). The development of the neomenid aplacophoran *Epimenia babai* occurs by unequal cleavage, and no indication of metamerism is observed. In the caudofaveate *Chaetoderma nitidulum*, early cleavage and early larval stages have not been studied, but it is now known this species exhibits epibolic gastrulation. Eight transverse dorsal ridges of late larvae may indicate structural homology with metameric polyphacophorans (Claus Nielsen, pers. comm.). It is expected that in the near future, additional detailed descriptions of early development will be described for new taxa. Both advanced microscopy and fluorescent lineage tracing techniques will greatly improve these observations.

Molecular developmental data

Homeobox genes (Hox): This gene family has been shown to have key roles in anterior-posterior patterning in bilaterian animals. These genes are usually organized in clusters and are usually expressed in co-linear patterns. Although just a few taxa representative from key protostome lineages have been sampled, the observations are consistent with findings from phylogenetic studies using 18S (de Rosa et al., 1999). Many phyla, including molluscs, are still poorly sampled and additional information is needed. The exception being putative homeobox domains identified in the heterodont bivalve *Donax texianus* (Adamkewicz et al., 2001), the abalone *Haliotis rufescens* (Degnan et al., 1993; Degnan et al., 1995), and in the limpet *Patella vulgata* (de Rosa et al., 1999). The expression of five *Hox* genes has been better characterized in *Haliotis asinina* to the trochophore through post-larval stages (Giusti et al., 2000; Degnan, 2001). Expression of most of these genes is usually restricted to the neuroectoderm as in annelids, interpreted as playing a role in the development of the central nervous system in lophotrochozoans. Two of these genes, however, are expressed in the mantle margin of the larvae suggesting involvement in shell formation. This expression pattern has been hypothesized as co-option of these *Hox* genes for a phyletic specialization in molluscs (Degnan, 2001). Expression studies in representatives from other molluscan classes will help in testing these potential new functions.

Otx and *otp*: *Orthodenticle/otx* and *orthopedia/otp* are homeobox gene families present in all bilaterians. Orthologs to these two genes (*Pv-otx* and *Pv-otp*) were cloned from *Patella vulgata* (Nederbragt et al., in press). The authors used the expression *Pv-otx* around the stomodaeum to argue in favor of homology of the larval mouth in bilaterians. In addition, *Pv-otx* is involved in the formation of ciliary bands, which is also found in other bilaterian taxa. Thus, this gene seems to maintain an ancestral function in multiple lineages. Finally, *Pv-otp* expression was found to be associated with the development of the larval nervous system, and in particular the larval apical sensory organ.

Engrailed: *Engrailed* is a homeobox gene shown to be involved in segmentation and neurogenesis in arthropods and annelids (Patel, 1994). Because some molluscs exhibit metamerism (i.e. polyplacophorans and monoplacophorans), this gene has been an early point of interest for evo-devo studies. Degenerate PCR in five different molluscan classes (Wray et al.,

1995) initially identified homologues of the gene. Expression studies by immunocitochemistry and in-situ hybridization, have been performed in representatives from four different classes: a) the chiton *Lepidochitona caverna* (Jacobs et al., 2001), b) the bivalve *Transennella tantilla* (Jacobs et al., 2001), c) the scaphopod *Antalis entalis* (Wanninger and Haszprunar, 2001), and d) the gastropods *Ilyanassa obsoleta* (Moshel et al., 1998) and *Patella vulgata* (Nederbragt et al., in press). In all cases, *engrailed* expression was associated with the embryonic shell gland in later embryonic stages or in larvae. Expression on early embryonic stages was examined in *Ilyanassa*, whereas only later stages were examined in the other taxa. Thus, results are not totally comparable and new comparative studies using similar experimental designs will likely be useful at understanding the role of *engrailed* in molluscan shell formation and synthesis.

Dpp-BMP2/4: In vertebrates and insects, these genes play a role in the specification of the dors-ventral axis. In *Patella vulgata*, however, the ortholog of this gene appears to have a different function being expressed in ectodermal cells surrounding the cells expressing *engrailed* (Nederbragt et al., in press). By comparing with other *dpp/engrailed* interations on other experimental organisms (i.e. *Drosophila*), the authors hypothesize an ancient involvement of *engrailed* and *dpp* in setting up compartmet boundaries between embryonic domains.

Snail and *Twist*: These genes are involved in mesoderm formation in chordates and ecdysozoans. In order to assess the function of *snail* in a member of the lophotrochozoan clade, Lespinet et al. (2002) studied the spacio-temporal expression of two *snail* homologues (*Pv-Sna1* and *Pv-Sna2*) in the gastropod *Patella vulgata*. Although some small mesodermal expression is present in *Patella*, the two homologues are mostly expressed in ectodermal derivatives. This expression pattern led the authors to propose a new ancestral function for *snail* in the bilaterian ancestor. In the case of *Twist*, this gene (*Pv-twist*) was shown to express in ectomesoderm of trochophore larvae in *Patella vulgata* (Nederbragt et al., in press), but not in endomesoderm. The authors suggested that other genes are likely involved in mesoderm formation and therefore the homology of this germ layer in bilateria still needs to be evaluated.

Esther32: *Esther32* has been identified as a putative RNA-binding protein in *Patella vulgata* (Klerkx et al., 2001). Spatio-temporal expression patterns show that this gene is not expressed in

cleavage-arrested or differentiated cells, which lead the authors to suggest that *Esther32* may be involved in maintenance of undifferentiated cells where expressed.

MAPK: As mentioned above, the D quadrant acts as an embryonic organizer for axial patterning in molluscs. The first attempt at uncovering the molecular mechanisms behind D quadrant specification has been performed in embryos from an unequal cleaver, the gastropod snail *Ilyanassa obsoleta* (Lambert and Nagy, 2001). Activation of the mitogen-activated protein kinase *(MAPK)* signal transduction cascade was found to be a key player in cell fate specification of early development in *Ilyanassa*. By inhibiting activation of the *MAPK* cascade in a time series, Lambert and Nagy (2001) demonstrated that a progressive specification of *MAPK* correlates with a progressive activation of cell fates. In the equal cleaver *Patella vulgata*, using a similar experimental procedure, Lartillot et al. (2002) were able to show that the inhibition of *MAPK* signaling also leads to an equalized cell division pattern in this limpet snail. Although alterations are more marked in *Patella* the role of the *MAPK* cascade in the regulation of cell cleavage appears to be conserved in gastropods.

Brachyury: A homologue to this gene *(PvuBra)* has been identified in *Patella vulgata* (Lartillot et al., 2002). *PvuBra* is expressed in the macromere (3D) as soon as its fate is determined, consequently spreading to neighboring cells that give rise to the posterior edge of the blastopore during gastrulation. *PvuBra* expression is maintained at posterior pole and along the developing anterior-posterior (AP) axis until the completion of gastrulation. This broad pattern of AP axis development induced by the activity of a posterior growth zone is observed across Bilateria, which lead the authors to suggest that *Brachyury* is a key component of a conserved developmental process already present in the bilaterian ancestor. Lartillot et al. (2002) successfully showed that *PvuBra* is a target of the *MAPK* signaling cascade in the macromere, proposing a new gene interaction that may be conserved across, at least gastropod molluscs, but most likely among more divergent lineages.

Hedgehog: Protostomes show a ventrally located nervous system, whereas deuterostomes have a dorsally located nervous system. There has been controversy whether these two systems are developmental homologues or not. *Hedgehog* plays a key role in midline patterning in

deuterostomes, and has recently been shown to express in the ventral midline ectodermal cells of the one-day old larva of *Patella vulgata* (Nederbragt et al., in press). These cells usually give rise to ciliated structures in molluscs, which in turn are thought to give rise to the nervous system. This observation, therefore lead the authors to conclude that *hedgehog* had an ancient role in the early patterning of the nervous system in the ancestral bilaterian.

Morphogenesis

One of the fascinating questions in molluscan body plans is the evolution of gastropod torsion, and many hypotheses have been proposed in order to explain this extreme anatomical modification. Larval musculature plays a key role in early torsion, and it is important to determine if larval muscles gives rise to adult muscle structures before models for the evolution of torsion can be improved. New higher-resolution techniques (i.e. SEM, TEM, spatio-temporal expression of tropomyosin, and phalloidin staining) have demonstrated that larval retractor muscles and the adult shell muscles are not homologous structures in basal gastropods (*Patella* and *Haliotis*) as previously thought (Page, 1997; Degnan et al., 1997a,b; Wanninger et al., 1999a,b).

Potential for genomic approaches

The availability of powerful molecular and genomic techniques has triggered an immense interest in comparative developmental biology. Most of these studies have included only model organisms developed initially as genetic systems (two ecdysozoans –the fruitfly and the nematode, and several vertebrates – zebrafish, frog, mouse and chick). Prior to the new animal phylogeny, *C. elegans* (a pseudocoelomate) was thought to be a representative of a lineage basal to coelomate bilaterians. Strong support for the ecdysozoan clade, which includes both the nematode and the fruitfly, leaves us with no lophotrochozoan organism represented in the modern developmental revolution. This clade, however, includes key phyla that exhibit extreme modification of their body plan, and Mollusca is one of the clearest examples. With several of the genomes from model organisms, either complete, or well underway, there is now room for the use of these modern approaches to understand the body plan architecture of non-model

organisms. Molluscs, because their diverse body plan architecture, are obvious candidates as targets of this new revolution.

It has now become clear that easy access to sequence data was simply a turning point in the whole process of understanding how organisms function and evolve. As new non-model organisms become targets of the new technological advances, we can predict that comparative thinking will have an even more fruitful impact than ever before, on the understanding of metazoan evolution.

CONCLUSION

Currently available molecular data suggest that molluscs belong to the lophotrochozoan assemblage. Although 18S alone has not provided enough resolution to resolve sister taxa relationships, data from new genes such as mitochondrial proteins and 28S may help address this question. In addition to sequence data, the few genomic characters available at present emerge as likely useful tools to study molluscan phylogenetics. Complete mitochondrial genomes will not only help in phylogenetic reconstruction, but also will provide insight into mechanisms of molecular evolution of this molecule in molluscan lineages. Other genomic signatures that appear to have potential as phylogenetic markers are ribosomal secondary structure and gene family evolution. Finally, evo-devo promises to be the merging ground of anatomy and molecules where we will be able to understand the interaction between the environment and the organism, and how the organism is shaped and regulated at the molecular level.

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Figure captions

Figure 1. a) The placement of the two aplacophoran subclasses in one clade with Polyplacophora as the sister taxon, traditionally been named Aculifera. b) The gradist hypothesis renders the Acuilifera paraphyletic, placing the two aplacophoran lineages at the base of the molluscan tree and Polyplacophora as the sister taxon to the crown lineages. The latter grouping is known as the Testaria.

Figure 2. Mitochondrial gene arrangements of lophotrochozoan and molluscan taxa. Genomes are graphically linearized at cox1. All genes are transcribed from left-to-right except those underlined to indicate opposite orientation. NCBI accession numbers depicted next to each genome. * In Kurabayashi and Ueshima (2000a). Gene designations: Cytochrome oxidase subunit I, II, III (cox1, cox2, cox3), cytochrome b (cob), NADH dehydrogenase subunits 1-6, 4L (nad1-6, 4L), ATP synthase subunits 6,8 (atp6, atp8), large ribosomal subunit (rrnL), small ribosomal subunit (rrnS), 18 transfer RNAs specifying a single amino acid (trnX), two transfer RNAs specifying leucine L_1=L(CUN) and L_2=L(UUR), two transfer RNAs specifying serine S_1=S(AGN) and S_2=S(UCN). (Modified from Boore. 1999). a) Comparison of three conserved lophotrochozoan mitochondrial genomes. b) Complete and partial molluscan mitochondrial gene arrangements.

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Figure 1

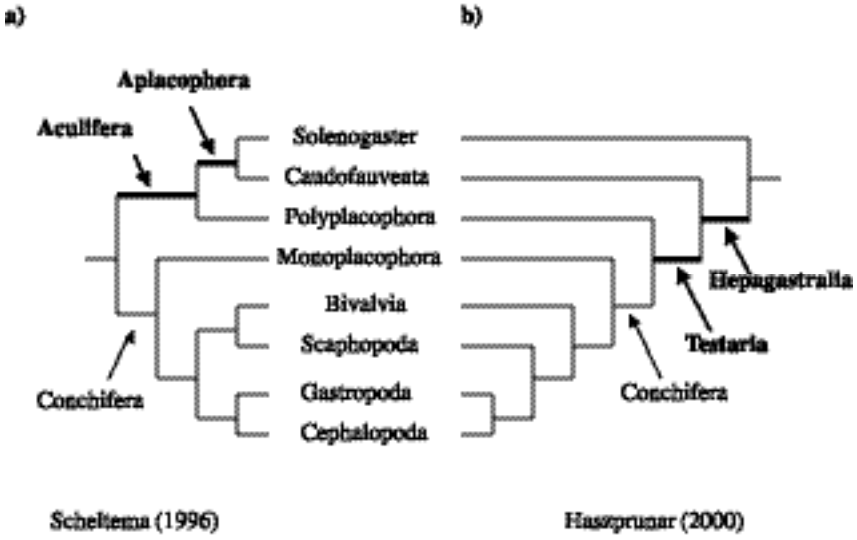


Figure2a

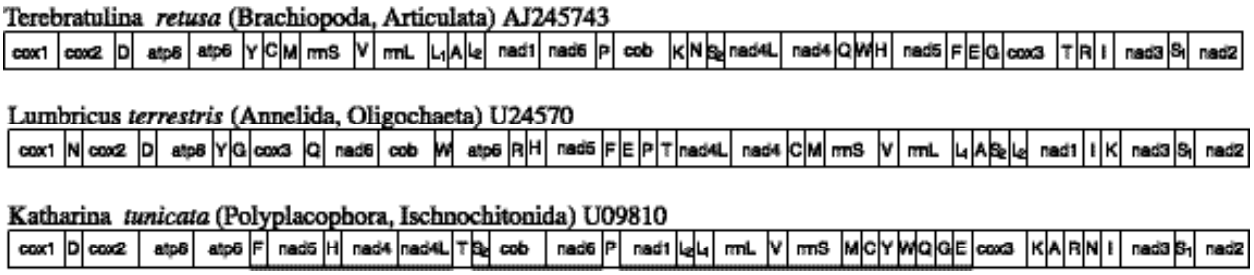
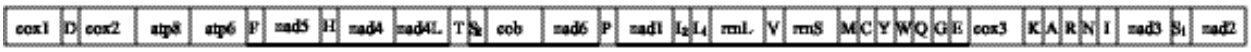
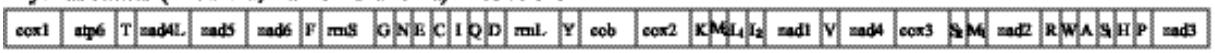


Figure 2b

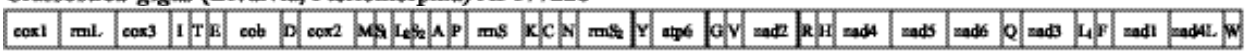
Katharina *tunicata* (Polyplacophora, Ischnochitonida) U09810



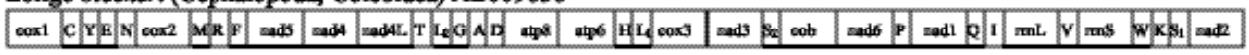
Mytilus *edulis* (Bivalvia, Lamellibranchia) M83756-62



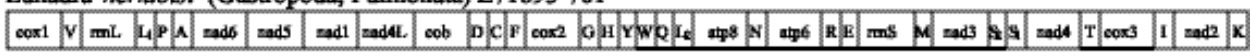
Crassostrea *gigas* (Bivalvia, Pteriomorphia) AF177226



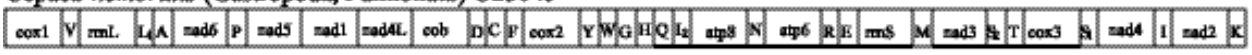
Loligo *bleekeri* (Cephalopoda, Coleoidea) AB009838



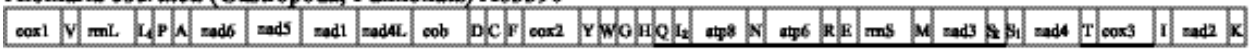
Euhadra *herkiotsi* (Gastropoda, Pulmonata) Z71693-701



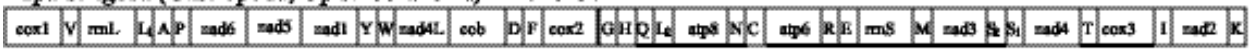
Cepaea *nemoralis* (Gastropoda, Pulmonata) U23045



Albinaria *coerulea* (Gastropoda, Pulmonata) X83390



Pupa *strigosa* (Gastropoda, Opisthobranchia) AB028237



Omalogyra *atomus* (Gastropoda, Allogastropoda) *

Littorina *saxatilis* (Gastropoda, Caenogastropoda) AJ132137